PROTEOLIPOSOMES AND ITS DERIVATIVES AS ADJUVANTS INDUCERS OF CITOTOXIC RESPONSE AND THE RESULTANT FORMULATIONS

DESCRIPTIVE MEMORY

The present invention is related to the vaccine field and its use in medicine. Particularly, it is related to the use of adjuvants and the resulting vaccine formulations.

The technique objective is to increase immune responses against fungal, viral, parasitic bacterial or cancer antigens, particularly to augment the induction of cytotoxic T-cell responses that are essential against these antigens. This approach will lead to the development of prophylactic or therapeutic vaccine formulations.

To achieve the aforementioned technique objective, it is proposed to use Proteoliposome and its derivatives as adjuvants in vaccine formulations containing fungal, viral, parasitic bacterial or tumour antigens, inserted in the proteoliposome structure as well as conjugated or mixed with these structures. These formulations would extend the preferential Th1 immune response induced by the Proteoliposome and its derivatives to the included antigens (Pérez O *et al.* Infect Immun. 2001, 69(7):4502–4508), inducing an immune response mediated by T cytotoxic lymphocytes against the antigen being administered by mucosal or parenteral routes or the combination of them.

STATE OF THE ART

The infection by intracelullar fungi, virus, parasites and bacteria are frequently cause of pathologies all over the world. Cancer is also a terrible scourge to the humanity. Those infections and cancer of human cells imply the production of high quantity of antigen in the cellular citosol, many of which are driven to the cellular surface associated to the molecules of the Major Histocompability Complex (MHC) class I (Heemels, M.-T. and H. Ploegh, 1995. Generation, translocation, and presentation of MHC class I-restricted peptides. Annu. Rev. Biochem. 64,463-491; Rock, K.L. 1996. A new foreign policy: MHC class I molecules monitor the outside world. Immunol. Today 17, 131-137; Jondal, M., R. Schirmbeck, and J. Reimann. 1996. MHC class I restricted CTL responses to exogenous antigens. Immunity 5:295-302; and Reimann, J. and S. H. E. Kaufmann. 1997. Alternative antigen processing pathways in anti-infective immunity. Curr. Opin. Immunol. 9:462-469)

The immune response has been phenotipically divided in cellular (Th1) and humoral (Th2). The Th1/Th2 patterns are distinguished firstly by the cytokines secreted by the T $CD4^+$ lymphocytes: mainly IFNγ and IL12 by Th1 and IL4 and IL5 by Th2 (Mossman, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A, and R. L. Coffman. 1986. Two types of murine helper T cells clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136:2348-2357). Besides, it is also possible to evaluate the type of induced response by determining the class and subclass profiles of serum immunoglobulins characteristics of each pattern of response. Thus, in mice the Th1 pattern induces mainly antibodies of the IgG2a subclass (dependent on IFNγ), whilst Th2 induces IgE and IgG1 subclass (dependent on IL4). In humans, the Th1 pattern induces mainly antibodies of the IgG1 and IgG3 subclass and the Th2 induces the IgE class.

Other cytokines produced by non-lymphoid cells have also been associated to these patterns of T-cell responses. This is the case of IL2 and IL18 that are associated to a Th1 pattern. Besides, IFNγ and IL10 act as inhibitors of the Th2 and Th1 response, respectively.

These classifications have been extended also to the T $CD8^+$ lymphosites which have been classified as Tc1 and Tc2 mainly based in cytokines production similarities. T $CD8^+$ lymphocytes are the main mediators and inductors of cytotoxic activity. These act then, not only as efector cells but also as regulatory cells developing one type of response more than other. Therefore, the determination of lymphocyte subsets T $CD4^+$ or T $CD8^+$ present in prolipherations of periferic mononuclear cells of immunized subjects or secondary lymphoid organs of immunized animals, re-stimulated *in vitro*, is an important evidence of the stimulation of this two populations.

It is necessary to eliminate tumour cells and cells infected with intracellular pathogens. The cytotoxic activity of T CD8+ lymphocytes (CTL) is one of the most efficient immune mechanisms in this respect. These lymphocytes recognize antigens presented in MHC-I context and are capable to kill the infected cells throught the liberation of perforins, production of granzymes and Fas-FasL interactions (O'Hagan D., Mackicham M., and Singh M. Recent advance in adyuvants for infection disease Biomolecular Engineering 2001). CTL activity is the immune response more commonly accepted to determine the effectiveness of vaccines against intracellular infections caused by virus, bacteria or protozoa, as well as against tumors.

CTL activity can be determined by any of the direct or indirect methods known preferably by combining them. Indirect ones determine if the Proteoliposome or its derivatives containing antigens inserted in their structure, conjugated or coadministered to them are phagocytosed, degraded and presented in the cellular surface of cell lines associated to MHC-I molecules. Those are then co-cultured with cytotoxic cell lines specific against the inserted antigen and the cellular death is determined by radioactive or not radioactive methods. Other indirect sign is the stimulation by the Proteoliposome or its derivatives containing antigens inserted in their structure, conjugated or coadministered, of T CD8+ lymphocytes in periferic mononuclear cells of immunized subject or secondary lymphoid organs of immunized animals, re-stimulated *in vitro* with the antigen of interest. The production of IFNγ and IL2 by T CD8+ lymphocytes present in mononuclear cells re-stimulated *in vitro*, could be other indirect way to evidence CTL activity and can be detected by Flow Cytometry or ELISPOT assay. Moreover, IL2 production by T CD8+ hybridoma cells after recognition of the specific antigen in the context of MHC-I molecules in the cellular surface of an antigen presenting cell previously incubated with the Proteoliposome or its derivatives containing the specific antigen inserted in their structure, conjugated or coadministered to them, is another indirect way to evidence CTL activity.In the direct methods to measure CTL responses, mammalians are immunized with the Proteoliposome or its derivatives containing antigens inserted in their structure, conjugated or coadministered to them. At times after immunisation, the secondary lymphoid organs are extracted to determine the presence of CTL activity against the specific antigen associated to MHC-I molecules. The oldest method to measure antigen specific CTL activity is the radioactive cromium release cytotoxic assay. It has been desinged for fresh cells (measuring effector CTL activity) as well as for CTL cell lines (measuring reactive CTL of memory). In both, the target cells express the specific antigen of interst. It can be achieved throught different methods (infection with a recombinant virus, peptide loading or genetic transfection).Recently specific CTL epitops previusly characterised are used.

Other *ex vivo* method is the tetrameric-binding assay (TBA). Tetrameric complex of MHC-I molecules charged with specif epitops bind directly the TCRs of antigen specific T CD8+ cells independently of its functional abilities. HLA-A2 tetramers are generaly used, due to its wide representation in the population.

CTL have been considered as the main element of the cellular response for a long time. Today it is known that not all Th1 responses strictly imply induction of CTL lymphocytes. Thus, it is necesary to find adjuvants capable to induce a Th1 pattern of response that includes CTL activity.

Adjuvants are substances that potentiate the specific immune response against an antigen causing a faster induction of it and augmenting its duration (Vogel FR. Dev. Biol. Stand. 1998,92:241-248). Its use in vaccine formulations allow to reduce the amount of necessary antigen, to direct the response towards the desired pattern as well as to minimise the number of necessary dose.

Among the available adjuvant systems that induce Th1 response are:

1. Monophosphoril lipid A (MPL), particularly MPL 3-D-O acetilated (3D-MPL) or other non toxic derivatives from lipopolisacharide (LPS) and combinations of MPL, preferably 3D-MPL or non toxic derivatives from LPS with aluminium salt.
2. Immunostimulatory fractions of *Quillaja* saponaria: Quil A incorporated with cholesterol and phospholipids in immunostimulatory complex ISCOM (Polakos NK, Drane D, Cox J, Ng P, Selby MJ, Chien D, O'Hagan DT, Houghtan M and Paliard X. J Immunol 2001,166:3589-98).
3. Particles of polilactil co-glicolidos (Putney SD and Burke PA, Nat BIOTECHNOL. 1998, 16:153-157).
4. MPL and saponin, particularly QS21 and 3D-MPL as is revealed in WO 94/00153.
5. QS21 and cholesterol as is revealed in WO 98/33739.
6. QS21, 33D-MPL and tocoferol emulsified in water and oil as is revealed in WO 95/17210.
7. Oligonucleotids containing no metilated sequences of CpG as is revealed in WO 96/02555.

Adjuvants inductors of CTL now a day are limited. Among them are the CpG, the QS21, the MPL, the ISCOM and the chocleates derivated from lipids (O'Hagan D., Mackicham M., and Singh M. Recent advance in adyuvants for infection disease. Biomolecular Engineering 2001 and Edelman Robert. The development and use of vaccine adyuvant. Molecular Biothecnology 2002. 21:2, 129-148.).

Proteoliposomes have been described for the preparation of prophylactic vaccines against infectious diseases by Ruegg CL *et al.* (Preparation of proteosome-based vaccines. J Immunological Methods 1990;135:101-109); Lowell *et al.* (Proteosome-Lipopeptide Vaccines: Enhancement of Immunity for Malaria CS Peptides. Science 1988;240:800-2) and also it was revealed in US No. 5,597,572. In the last one, the main core is a proteoliposome derivated from the external membrane of *Neisseria meningitidis* serogroup B, whose particulate structure, the content of native LPS incorporated and not free, the presence of polysaccharide from *Neisseria meningitidis* serogroup C, its lipidic composition and its adsorption in aluminium, are related to its high immunogenicity and probed protection in humans.

A vaccine based on this proteoliposome, VA-MENGOC-BC™, has been applied in more

than 50 millions of doses demonstrating to be safe, non reactogenic and effective to protect against *N. meningitidis* serogroups B and C. Is also applied during the breastfeeding period in a safe and effective way, turning the polysacharide C from T independent to T dependent antigen (Pérez O. et al. Th1 response induced by the B component of VA-MENGOC-BC™ overcomes the thymus independence of polysaccharide C and primes for memory in toddler. Biotecnología Aplicada 2002; 19(1-2):54). This vaccine induces a preferential Th1 pattern of response characterized by the induction in humans and animals of lymphoproliferation; anti-Proteoliposome IgG antibodies; IgG1 subclasses in human and IgG2a in mice; IFNγ, IL-2 and IL-12 at mRNA and protein levels. Besides, this vaccine neither induces IgE antibodies anti-Proteoliposome nor increases the level of total IgE antibodies. Also this vaccine does not induce production of IL-4 and IL-5 neither as protein nor at the level of mRNA (Pérez O *et al.* Infect Immun. 2001, 69(72001):4502–4508). The proteoliposome and its derivatives as VSSP and AFCo1, have been also used as adjuvants as revealed in (Método de obtención de estructuras cocleares. Composiciones vacunales y adyuvantes basados en estructuras cocleares y sus intermediarios. OCPI. 2002-0292 of 27/11/02) and in (US 6,149,921 of 2000), respectively.

The mechanisms of protection against *Neisseria* (extracellular Gram negative bacteria) are related to the induction of antibodies that mainly mediate bactericidal and opsonophagocitic functions. Thus, it may appear unlikely that the proteoliposomes derivated from these bacteria may induce CTL responses.

The present invention has the objective to employ bacterial proteoliposomes especially those derived from *N. meningitidis*, like AFCo1, as new adjuvants inducers of CTL response. These new adjuvants are of particular importance against fungal, viral, parasitic and bacterial infections (mainly intracellular) as well as cancer.

"Proteoliposome" means that they are obtained from bacterial strains by using any know methos as: its isolation without detergent, a process including detergent (as desoxicholate) or extraction from vesicles present in culture supernatants as is particularly revealed in US 5,597,572. Proteoliposomes contain different patogen associated molecular patter (PAMP) capable to strongly stimulate to the immune system.

"AFCo1" means adjuvants derived from Proteoliposomes mainly from bacterial origin that also contain PAMP as is revealed in the patent (Método de obtención de estructuras cocleares. Composiciones vacunales y adyuvantes basados en estructuras cocleares y sus intermediarios. OCPI. 2002-0292 of 27/11/02).

Laboratory studies have surprisingly demonstrated, that humans immunized with VA-MENGOC-BC™, anti- *N. meningitidis* B and C vaccine, show activation and proliferation of T $CD4^+$ and T $CD8^+$ lymphocytes against the proteoliposome after restimulation *in vitro* of periferic blood mononuclear cells (PBMC).

Also, it has been surprising to find that Balb/c mice immunized with VA-MENGOC-BC™ or Proteoliposomes or AFCo1 mount proliferative T $CD8^+$ lymphocytes responses besides the known T $CD4^+$ responses.

Also it has been unexpectedly found, that the natural infection with the microorganism *N. meningitidis* serogroup B induces T $CD4^+$ as well as T $CD8^+$ response against Proteoliposome. These responses can be detected in lymphocytes from individuals cured from meningoccocal desease caused *N. meningitidis* B.

It was also unexpected that also mice inoculated with *N. meningitidis* B mount T $CD8^+$ lymphocyte responses.

It has been demonstrated that T $CD4^+$, and surprisingly T $CD8^+$ lymphocytes from humans immunized with VA-MENGOC-BC™ as well as from individual cured from meningoccocal desease secreted IFNγ and IL-2 but neither IL-4 nor IL-5. This was evaluated by Flow Cytometry. These results include T $CD8^+$ lymphocytes amongst the cells able to produce cytokines characteristics of the Th1 pattern of response, evidencing that CTL responses are being functionally induced.

The incorporation of exogenous antigens in the Proteoliposome and its derived has been efficiently performed with the inclusion of Ovoalbumin (OVA), proteins of *Leishmania* and other PAMP as LPS. It would be possible to incorporate other antigens in the proteolyposome structures. The AFCo1 derived from Proteoliposome can also efficiently incorporate nucleic acids, especially plasmids.

Proteoliposomes that could express modified concentrations of the antigen of interest, throught the manipulation by genetic ingeneering of the producing cell with the objective that it expresses more or less quantity of the desired antigen is also aimed by this invention.

"Antigen of interest" means those from fungi, virus, parasites, bacteria and cancer cells that require CTL responses for their efficient elimination by the immune system, without any restriction to the antigens already identified. "More or less" means specifically that the strain expressess more than 20, 15, 10, 5, 4, 3, 2, 1, 0.5 ó 0.1% of the quantity of the antigen or the antigens of interest. Preferably the modified strain by genetic engineering express from 0.5 to 10% of the antigen of interest.

The gene encoding for the antigen of interest of the invention can be modified by genetic ingeneering by known techniques. Particularly the meningoccocal strain can be genetically altered as is revealed in the patent WO 01/09350 or any other method.

It is also part of this invention the obtention of different vaccine formulations exploiting Proteoliposome and AFCo1 abilities to induce CTL response, non previously described.

The effectiveness of these formulations with antigens incorporated in the proteoliposomes or in the AFCo1 being inserted in the lipidic bilayer or trapped in their structure has been demonstrated. Also have shown to be effective vaccine formulations where the antigens are conjugated to a part of the Proteoliposome. Another effective possibility is to transform them in choclear structure like AFCo1 after conjugation.

The vaccine formulations of the present invention can be used to protect a mammalian susceptible to an infection or to treat tumoral diseases by administering them systemically or mucosally. These applications may include intramuscular, intraperitoneal, intradermic, or subcutaneous injections or mucosal administrations by oral/feed or nasal/respiratory routes or genitourinary tract.

The amount of Proteoliposome or AFCo1 in each formulation is selected as the amount that shows adjuvant function, being always less than the used in the anti-meningoccocal vaccines based in these structures, it imply that that the secondary effect are minor and not significant. This amount can vary depending of tha antigent of interest and the way of its incorporation. Generally the amount of Proteoliposome or AFCo1 will be among 1 and 50 µg per dose and more typically among 5 and 25 µg. The amount of the antigen of interest will be in the rank of 0.1 to 20% of the Proteoliposome or AFCo1 mass and preferably 0.5 to 10%.

The number of doses will depend firstly on the type of formulation, prophylactic or therapeutic. In the fist one, a maximum of three doses will be applied and in the second

one, up to five doses can be applied. Both can be applied in childen under one year of age, from 2 to 5 years, schoolchildren, adolescent, adults and elderly persons. The innOvation of this invention is the use of the Proteoliposomes or AFCo1 derived from outer membran of Gram-negative bacterias and particularly from *N. meningitidis* B, as adjuvants inductors of CTL activity.

It is particularly novel that the natural infection with *N. meningitidis* B, extracellular bacteria, induced response of T $CD8^+$ cells, lymphocytes associated with cytotoxic activity, also it was surprinsing that they produced IFNγ and IL-2, evidencing the induction of CTL activity. Thus, these lymphocytes are increasing the Th1 response previously described.

Other novel aspect is its application in prophylactic formulations against fungal, viral, parasitic and bacterial infections and its applicatons in therapy of subjects affected by malignant tumors to induce citotoxic response.

It is particularly novel, the conjugation or insertion of cytotoxic epítopes in the Proteoliposomes or AFCo1 increasing the CTL response.

It is novel the flexibility of the AFCo1 to include particulate antigens, especially DNA.

The possibility to use these formulations by the mucosal route, besides the parenteral route and the combination of both is also novel.

The modification by genetic engeneering of meningoccocal strains to produce Proteoliposomes or its derivatives expressing cytotoxic antigens is another novel aspect of this invention.

The proposed solution has the following advantages:

The immunologic effect achieved by these formulations allowed the induction of CTL response by the Proteoliposome or the AFCo1 against the antigen of interest included on it. This is applicable but not restricted to intracellular microorganism and tumor diseases.

The immunological effect of the Proteoliposome as vaccine and transitively as adjuvant, where lower concentrations are used, is safe in children younger than 1 year, from 2 to 4 years, schoolchild, adolescents and adults.

The Proteoliposomes and the AFCo1 also turn T-independent antigens as carbohidrates (conjugated or cOvalently included) into T-dependent antigens due to the preferential cellular response (Th1) induced by them. This property is also applicable to antigens of interes of sacaridic nature.

The present invention will be described through the following specific examples.

Example 1. Proteoliposome obtaining. For obtaining Proteoliposome a culture of *N. meningitidis* B or *Salmonella typhi* is performed and the biomass collected by centrifugation is subjected to a process of extraction with detergents, enzymes and ultrasound. Cellular debris is removed through centrifugation and the supernatant is subjected then to a digestion with nucleases to eliminate nucleic acids. The extract is recovered through ultracentrifugation, resuspended in a solution with detergent and purified from the rest of components of low and medium molecular weight throught a cromatography of molecular exclusion. The Pretroliposome obtained contain less than 10% of nucleic acids and less than 10% of inserted LPS in its structure but never free. The LPS is essential as danger signal to trigger the innate immune response. The final product is subjected to a group of biological and physico-chemical controls.

Example 2. T CD4$^+$ and T CD8$^+$ lymphocytes response in immunized mice. Balb/c mice were immunized with two doses of Proteoliposome or AFCo1, 25 μg each one, by intramuscular or nasal routes 14 days apart. Spleen cell were isolated 7 days after the second dose and incubated with the Proteoliposome. The expanded cells were treated with anti-CD4 or anti-CD8 monoclonal antibodies and subsecuently labelled with anti-IgG to be evaluated by Flow Cytometry. The immunization stimulated both T CD4$^+$ and T CD8$^+$ lymphocytes anti-Proteoliposome. 45% of lymphocytes were T CD4$^+$ and 40% were T CD8$^+$.

Example 3. T CD4$^+$ and T CD8$^+$ lymphocytes response in immunized human.

Young adults were immunized with two doses of VA-MENGOC-BC™, 50 μg each one, 6 weeks spaced. PBMC were purified over Ficoll-Hipaque from blood sample taken 7 days after the second dose. The expanded cells were treated with monoclonal antibodies anti-CD4 or anti-CD8 and subsequently labelled with an anti-IgG to be evaluated by Flow Cytometry. The immunization stimulated both T CD4$^+$ and T CD8$^+$ lymphocytes anti-Proteoliposome. 50% of lymphocytes were T CD4$^+$ whiles 42% were T CD8$^+$.

Example 4. T CD4$^+$ and T CD8$^+$ lymphocytes response in convalescents from meningoccocal desease. PBMC were purified over Ficoll-Hipaque from blood sample taken from indivuduals convalescents from meningoccocal desease caused by *N. meningitidis* B two moths after be discharged. Those were incubated 4 days with the Proteoliposome. The expanded cells were treated with anti-CD4 or anti-CD8 monoclonal antibodies and subsequently labelled with anti-IgG to be evaluated by Flow Cytometry. The immunization stimulated both T CD4$^+$ and T CD8$^+$ lymphocytes anti-Proteoliposome. 47% of lymphocytes were T CD4$^+$ and 39% were T CD8$^+$.

Example 5. T CD4$^+$ and T CD8$^+$ lymphocytes response in human which carries *N. meningitidis*. PBMC were purified over Ficoll-Hipaque from blood sample taken from individuals which carries *N. meningitidis* B, previously identified by nasopharingeal exudates. Those were incubated 4 days with the Proteoliposome. The expanded cells were treated with anti-CD4 or anti-CD8 monoclonal antibodies and subsecuently labelled with anti-IgG to be evaluated by Flow Cytometry. The immunization stimulated both T CD4$^+$ and T CD8$^+$ lymphocytes anti-Proteoliposome. 35% of lymphocytes were T CD4$^+$ and 32% were T CD8$^+$.

Example 6. Activation signals and intracellular cytokines production by lymphocytes from immunized humans.

Young adults were immunized with two intramuscular doses of VA-MENGOC-BC™, 50 μg each one, 6 weeks apart. Samples of bood were taken few months after the second dose. These were incubated with Proteoliposome at 10 or 20 μg/ml for 27 hours. Later, red cells were lysed and the other cells were treated to become permeable to monoclonal antibodies for detection of intracellular cytokines (IFN-γ, IL2 or IL5) and surface markers (CD4, CD8 or CD69). 3.28% of T CD4$^+$ and 20.65% T CD8$^+$ lymphocytes were activated with Proteoliposome 10 μg/ml while 3.86% T CD4$^+$ and 14.11% T CD8$^+$ lymphocytes were activated with Proteoliposome 20 μg/ml. Nevertheless, just activated T CD8$^+$ produced IFN-γ and IL-2, although a moderated production of IL-2 was also detected in activated T CD4$^+$. 0.35% T CD4$^+$ and 0.89% T CD8$^+$ lymphocytes produced intracellular IFN-γ after incubation with Proteoliposome at 10 μg/ml while 1.54% T CD4$^+$ and 1.29% T CD8$^+$ lymphocytes produced it after incubation with Proteoliposome 20 μg/ml. However, just 0.57% T CD8$^+$ producing IFN-γ lymphocytes was activated after stimulation with Proteoliposome at 10 μg/ml and 0.87% after incubation with Proteoliposome at 20 μg/ml.

The percentages of lymphocytes producing intracellular IL-2 were 1.19% T $CD4^+$ and 1.07% T $CD8^+$ after stimulation with 10 μg/ml of Proteoliposome and 1.18 % T $CD4^+$ and 1.6% T $CD8^+$ after stimulation with Proteoliposome at 20 μg/ml. However, just 0.68% of T $CD8^+$ producing IL-2 lymphocytes were activated after stimulation with Proteoliposome at 10 μg/ml and 0.7% after incubation with Proteoliposome at 20 μg/ml. In the case of T $CD4^+$ lymphocytes just a low percentage of activated cells producing IL-2 were detected.

Example 7. Activation signals and intracellular cytokine production by lymphocytes from patients convalescent from meningoccocal desease. Blood samples were taken from indivuduals convalescent from meningoccocal disease caused by *N. meningitidis* B two months after the patients were discharged. These were incubated with Proteoliposome at 10 μg/ml for 27 hours. Later, red cells were lysed and the other cells were treated to become permeable to monoclonal antibodies for detection of intracellular cytokines (IFN-γ, IL2 or IL5) and surface markers (CD4, CD8 or CD69). 9.29% T $CD4^+$ and 26.28% T $CD8^+$ lymphocytes were activated. Both subsets of activated lymphocytes produced IFN-γ and just the T $CD8^+$ produced IL-2. 3.46% T $CD4^+$ and 1.39% T $CD8^+$ lymphocytes produced intracellular IFN-γ after incubation with Proteoliposome at 10 μg/ml but just 0.65% T $CD8^+$ and 0.95% T $CD4^+$ producing IFN-γ lymphocytes were activated. The percentages of lymphocytes producing intracellular IL-2 were 0.73% T $CD4^+$ and 0.93% T $CD8^+$ after stimulation with 10 μg/ml of Proteoliposome but just 0.73% T $CD4^+$ and 0.27% T $CD8^+$ producing IL-2 lymphocytes were activated after stimulation with Proteoliposome 10 μg/ml. Production of IL-5 was not detected.

Example 8. Inclusion of exogenous antigens in the Proteoliposome. Ova was included in the Proteoliposome throught the use of detergents, particularly deoxycholate, which allowed the disruption of the proteoliposomic structure. Detergent elimination resulted in re-assembly of the proteoliposomic structure in the presence of Ova in a proportion of 100:11.2. Proteoliposomes entrapping Ova were purified by gel filtration chromatography. The proteoliposomic structure was confirmed by their chromatography profile that was similar to the one of untreated Proteoliposomes. Also, SDS-PAGE and Western blotting using Ova specific polyclonal serum confirmed the presence of Ova in the Proteoliposomes. The amount of encapsulated Ova was estimated by densitometry analysis on polyacrilamide gels stained with Coommassie blue using an ImageMaster®VDS.

Example 9. Inclusion of exogenous antigens in AFCol. The inclusion of proteins from promastigotes and amastigotes of *Leishmania* was efficiently performed during the process of formation of AFCol. The immunization in animals of AFCol containing these antigens evidenced an increase of the anti-*Leishmania* immune response and the reduction of the indurations produced by the infection with this protozoa. The presence of these proteins included in AFCol was evidenced by SDS-PAGE followed by Western Blot revealed with anti *Leishmania* antibodies.

Example 10. Conjugation of antigens to the Proteoliposome. This is described in the Example 1.2 of the patente "Método de obtención de vacunas conjugadas. Composiciones vacunales" Patente OCPI 2002-0257 de 14/11/02.

Example 11. Inclusion of PAMPs. LPS from *Neisseria meningitidis* B have been included in different quantities, increasing the normal concentration of it in the Proteoliposome up to 12%. It has increased the immune response obtained against the Proteoliposome. LPS from *Vibrio cholerae* have been also included in the Proteoliposome inducing increased immune response against this antigen.

Example 12. IFN-γ Production by T $CD8^+$ lymphocytes against antigens included in or conjugated with Proteoliposomes or AFCo1. Spleen cells from animals immunized with Proteoliposomes including Ova or AFCo1 including *Leishmania* proteins were isolated. Induction of T $CD8^+$ lymphocytes population was evidenced as well as their ability to produce IFN-γ determined by ELISPOT T assay. The conjugates of polysacharyde C to the Proteoliposome induced IFN-γ determined by ELISA.

Example 13. Inclusion of plasmid DNA in the AFCo1 and its delivery in the cellular cytoplasm. The plasmid pGFP, encoding the green fluorescent protein under an expression promotor from superior cells (CMVp), was efficiently included in AFCo1. The AFCo1 containing this plasmid was added to a culturo of L929 cell line. The expression of the green fluorescent protein in the cellular cytoplasm was evidenced through an optic fluorescence microscopi.

Example 14. Dendritic cells exposed to Proteoliposome (PL) containing Ovalbumin (Ova) can present Ova peptides to T-cells. MHC class II restricted $CD4^+$ or MHC class I restricted $CD8^+$ T-hybridoma cells that are specific for Ova (257-264) and Ova (265-277) peptides, respectively were used. Dendritic cells (DC) were pulsed for 2 h with PL-OVA or soluble OVA and then co-cultured with T-cell hybridomas. Supernatants were collected after 24 h and tested for IL2 production using [^{3}H]thymidine incorporation by the IL2-dependent CTLL cell line. IL2 production was used as a measure of the activation of the Ova-specific cell hybridomas. Figure 1 shows that DC pulsed with PL-Ova can present Ova antigen to both $CD4^+$ and $CD8^+$ T-cell hybridomas. T-cell hybridomas produced significantly higher levels of IL2 when stimulated with DC that had been pulsed with PL-Ova as compared to DC pulsed with Ova alone. Significant differences between the levels of presentation of specific Ova peptides in MHC-I and MHC-II by DC incubated with PL(Ova) or Ova were determined by Duncan's multiple comparison test with 95% confidence level. Thus, Proteoliposome can deliver antigens to DC for efficient antigen presentation to T-cells.